ISOLATION AND MOLECULAR IDENTIFICATION OF BRUCELLA MELITENSIS IN ABORTED FETUSES OF SHEEP AND GOATS IN SULAIMANI PROVINCE, IRAQ

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ABSTRACT

Background

Brucellosis is a zoonotic disease that is endemic in Sulaimani Province, Iraq. The most accurate, credible, and susceptible method for detecting Brucella DNA is Polymerase Chain Reaction (PCR). A significant increase in abortion rates among the sheep and goats population in Sulaimani Province, Iraq was recorded by Veterinary centers.

Objectives

To detect the most common causes of abortion in sheep and goats.

Materials and Methods

A total of 73 aborted fetuses were collected during the delivery season in 2018, 53 sheep and 20 goats. Four sets of primers gene were achieved: firstly, for identification of the positive infection with all possible *Brucella* genus, the universal 'catch-all' primers (OMP2). Others, the specific primers (IS711) for identification *Brucella* species in all positive and negative samples.

Results

PCR showed that Brucella was the important cause of abortion in sheep and goats ($P<0.05$). *Brucella melitensis* was the only species detected, with prevalence rates of (32%) and (60%) in sheep and goats, respectively. Some independent variable risk factors were estimated that were statistically significant, including sex and age of the fetus, age of dam, infected organs of the fetus, history of abortion, and parity.

Conclusion

There was a significant relationship between abortion and the history of abortion in sheep and goats. Brucella melitensis is a serious reason for abortion in sheep and goats, and a great hazard to public health in Sulaimani Province.

Keywords: *Brucella detection; PCR technique; Isolation, Aborted fetus.*

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INTRODUCTION

Brucellosis is one of the most prevalent bacterial zoonotic diseases and causes considerable economic losses due to abortion in farm animals; additionally, it is possibly a serious multi-system infection in humans [1]. *Brucella melitensis* is the most common species that cause abortion in sheep in Iraq [2]. It was reported that this bacteria is the most common zoonotic bacteria in Samawa city- Iraq [3].

Brucella species are the most important agent of food-borne diseases that infect humans by ingestion of infected dairy products from sheep, goats, and cattle with Brucella types[4]. Recently brucellosis has emerged as major public health and economic problem in numerous countries of the Middle East including Saudi Arabia, Iraq, Lebanon, Kuwait, Yemen, and Jordan[5]. Brucellosis is noticeably high in Syria, Saudi Arabia, Iran, and Iraq [6]. The most virulent of the *Brucella* genera is *Brucella melitensis* and it has three biovars, with biovar 1 and 3 being the most frequently isolated forms in small ruminants in the Mediterranean area, the Middle East, and Latin America[7].

The most visible clinical manifestation in animals is spontaneous abortion, whilst weak or stillborn lambs and low milk production are other clinical observations. Large numbers of the pathogen remain vital in aborted tissues and inappropriate soil conditions. The infection results from direct contact with membranes and secretions, in addition to birth tissues expelled from infected animals [8]. *Brucella* species are stable in the environment as well as being gram-negative coccobacilli, which are transmitted through various routes including direct animal or environmental contact, consumption of raw or poorly cooked animal products, and aerosols[9] . Brucellosis is known as the most common zoonosis globally, with over 0.5 million new infections reported annually [10]. The classic and natural host of *Brucella melitensis* are goats and sheep [11].

The popular practice is for grazing sheep and goats to share food and water sources before coming back to their pens. The mixing of animals is an impact factor in the spread of infection with *Brucella* from infected to healthy animals and leads to difficulties in control [12]. In a lot of affected countries, brucellosis may cause infection in humans, domestic animals, and some wild animals and marine warm-blooded animals [13]. Indeed, Brucellosis is the second most critical zoonosis after rabies worldwide [14].

Genotyping of *Brucella melitensis* is vital for follow up of epidemiological observation in areas where it is endemic among domestic animals [15]. PCR assay is increasingly used as a high-confidence, cost-effective, sensitive, specific, and rapid method to identify the specific sequences of the *Brucella* genus, species, and biotypes and is also used as a surveillance tool to decrease outbreaks of brucellosis[16]. The increase of the geographical area of brucellosis is continually altering, with new foci emerging or re-emerging [15]. The goal of the current study is to investigate the main causative agents of abortion in sheep and goats by culture technique and PCR. This detection is very impactful to control futures and the treatment of the disease.

MATERIALS AND METHODS

Study design and sample collection

During the birth seasons from October 2018 to February 2019, a total of 73 aborted fetuses (53 sheep and 20 goats) of local breed non-vaccinated sheep and goats were collected from different flocks in Sulaymaniyah province Iraq. Post-mortem examinations were carried out on the abomasal content, liver, and spleen of the aborted fetuses.

Variable risk factors including frequency of abortion, sex, age of the fetus, age of dam, and parity were obtained using a questionnaire data form. The fetuses' ages were evaluated according to crown-rump length (CRL) [17].

The collected samples from the aborted fetuses were delivered to the College of the Veterinary Medicine Research Center University of Sulaymaniyah in a cold container. Autopsy samples were taken from the spleen, liver, and abomasa and stored in 7°C labeled sterile Epindorf tubes.

Isolation of pathogens

All aborted samples were processed inside a class III biological safety cabinet [9]. Tissue specimens were first decontaminated using ethanol and a flame and then homogenized in a sterile tissue grinder with a small amount of normal saline. Autopsies were prepared for isolation of *Brucella* according to modified Farrells serum dextrose agar according to standard procedures [18, 19]. Specimens obtained from liver, spleen and fetal

stomach content of all referred aborted fetuses were cultured on Brucella agar (Himedia, India) containing 5% (v/v) inactivated horse serum, 5% (w/v) dextrose, and Brucella selective supplement (Oxoid, UK) according to the manufacturer's instruction. Brucella is fastidious and relatively slow-growing organisms; hence, clinical samples are often grossly contaminated.

Plates were inoculated with sample material and incubated aerobically and in the presence of 5–10 % carbon dioxide at 37 °C. These plates were examined 3–7 days post-inoculation for bacterial growth [20]. Based on morphology, biochemical and microscopic examinations, suspected colonies were further subculture for the next one more week to obtain pure isolated colonies. Besides, these suspected colonies were subcultures on *Brucella* agar slopes for further evaluation and molecular identification.

Primer design and PCR Amplification

A DNA extraction kit (Geneaid Biotech Ltd. Taiwan) was used for DNA extraction from isolated colonies, and then amplification was carried out using conventional PCR master mix (Geneaid Biotech Ltd. Taiwan) according to the manufacturer's instructions without modification.

Four different sets of primers were designed for DNA amplification (as specified in table 1, forward and reverse primer sequences). Oligonucleotide primers specific for Brucella species were used to amplify the insertion sequences OMP2 856bp (in this study) and Oligonucleotide primers specific for *B. melitensis* IS711 252 bp, *B. abortus* IS711 113 bp(21), and *B. suis* IS711 285 bp [22]. The sequences of the primers and their lengths are listed in Table 1.

Amplification reactions were performed using a DNA thermocycler (Techne Prime UK) starting with an initial incubation at 94ºC for 2 minutes followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 Seconds, elongation at 72ºC for 60 Seconds, and then a 10 minutes final extension at 72ºC by only 1 cycle.

A 10 µl aliquot for each reaction mixture was then subjected to electrophoresis in1.5% agarose gel containing ethidiom bromide. Gels were visualized under UV light and documented using the usual visualization machine.

A 6–10-µl aliquot of each reaction mixture was subjected to electrophoresis in

1.5% agarose gel containing ethidium bromide. Gels were visualized under UV light and documented using Uvitec System DOC-008.XD (EEC, UVItec Ltd, Cambridge,

UK). A molecular weight marker with 100-bp increments (100 bp plus ladder Gene Ruler Fermentas International Inc., Canada) was used as size standards The expected product size was 864 bp for the *Brucella* genus and 252 bp for *Brucella melitensis* species. *B. melitensis* vaccine strain Rev.1. (JOVAC JORDAN BIO INDUSTRIES CENTER) and Master Mix without DNA were used as positive and negative controls, respectively. The PCR products of positive *Brucella* samples were then subjected to Sanger sequencing (Macrogene.Inc. South Korea).

Ethics statement

This study is approved by the Scientific and Ethical Committee of the College of Veterinary Medicine, University of Sulaymaniyah, Kurdistan Region, Iraq.

Statistical analysis

A multivariate logistic regression method was used for statistical analysis of the data. Before applying logistic regression, the relationship between each independent variable with infection was analyzed using the Chi-square test. The 95% confidence interval for the prevalence of bacterial infection was calculated using binomial distribution. GenStat 12 was used for statistical analysis. The significant level was considered $P < 0.05$.

Table 1. The Oligonucleotide primers used to amplify the target genes

Target species	Primers	Sequences (5'-3')	Length	Referecne
Catch-all	*omp2-F*	5'GGC AGC CTT CTG CGT AGC CTG3'	856bp	In this study
	omp2-R	5'GCT CTG GTT GCA GCT TCC GGC3'		
Brucella melitensis	*IS711-F*	5'CAT GCG CTA TGT CTG GTT AC3'	252bp	(22)
	IS711-R	5' AGTGTTTCGGCTCAGAATAATC 3'		
Brucella abortus	*IS711-F*	5'CAT GCG CTA TGT CTG GTT AC3'	113bp	(22
	IS711-R	5GGC-TTT-TCT-ATC-ACG-GTA-TTC3'		
Brucella suis	*IS711-F*	5'GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG3'	285bp	(23)
	IS711-R	5'TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT3'		

RESULTS

1. Isolation of bacteria

In the present study, liver, spleen and abomasal contents of 73 aborted sheep and goat fetuses were used for culturing and isolation. All cultures were also confirmed by conventional PCR. Positive isolation for *Brucella* in sheep and goats were 17 (32.07%) and12 (60%) samples, respectively. If positive results were obtained from one or more of the animal's organs the fetus was considered as positive (Table 2).

2- Molecular detection

All isolated bacteria from aborted sheep and goat fetuses by culture were also confirmed for *B. melitensis* by PCR (Table 2). All molecular detected positive samples were *B. melitensis*, with no detection of *B.abortus* or *B. suis* (Figure 1, 2). In sheep, the positive from autopsy samples were found in abomasa, liver and spleen at rates of 8(5%), 5(9.4) and 4(7.5), respectively. In goats, the highest rates were also recorded in the abomasa, 6(30%), followed by the liver 2(10) and spleen 4[20] (Table 2).

3-Risk factors evaluation

Four variable conditions were analyzed in this molecular study including the history of abortion, sex, age of the fetus, age of dam, and parity. In the history of abortion for sheep (57.1%) had a previous abortion but in the goats76.9% didn't have a history of abortion. The male percentage was (83.3%) in goats. Most infected aborted fetuses in sheep and goats were more than 5 months age or more. The higher rate of infection was in the first parity. The abomasa were found to be a highly significant risk factor when compared with other organs by an odds ratio of 7 and P< 0.05. In sheep with liver infection, in terms of correlation with the history of abortion, a highly significant relationship was indicated by P <0.01. Moreover, the same correlation at P <0.01 was detected in goats with a liver infection (Table 4).

A highly significant relationship was also reported in goats with infected abomasa at P <0.01, whilst in sheep with infected spleens, an odds ratioof5.7was found to be a considerable risk factor for a history of abortion. Overall, the data revealed that the history of abortion in sheep and goats is a serious factor in abortion but other factors show no connection with an infection of the fetus (Table 4).

Table 2. Isolation and PCR detection of Brucella melitensis species in aborted fetal organs.

Fetus organ	No.	Positive Culture (%)	PCR +Ve (*B. melitensis*)	No.	Positive Culture (%)	PCR+Ve (*B.melitensis*)
Liver	53	4(7.5)	4(7.5)	20	4(20)	4(20)
Spleen	53	5(9.4)	5(9.4)	20	2(10)	2(10)
Abomasa	53	8(15.09)	8(15.09)	20	6(30)	6(30)
Total	53	17(32.07)	17(32.07)	20	12(60)	12(60)

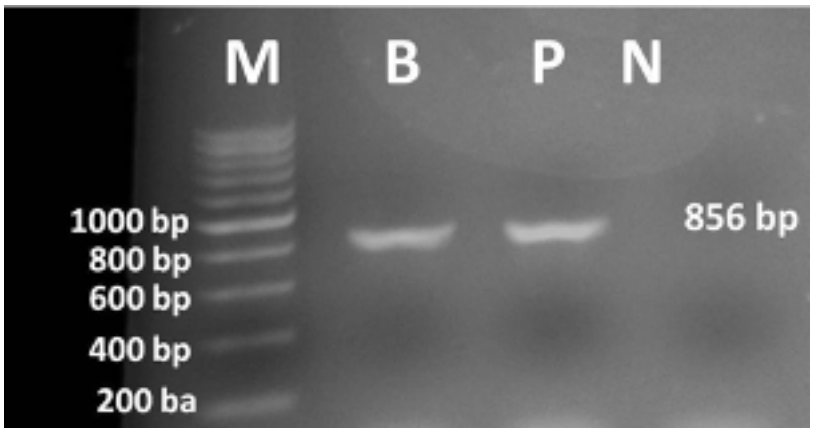

Figure1. Agarose gel electrophoresis of PCR assays products. Lane M: 200 bp DNA ladder (ROTH); lanes B: DNA of Brucella genus from the aborted fetus (856 bp); lane P: positive control DNA of *Brucella melitensis* vaccine; lane N: negative control.

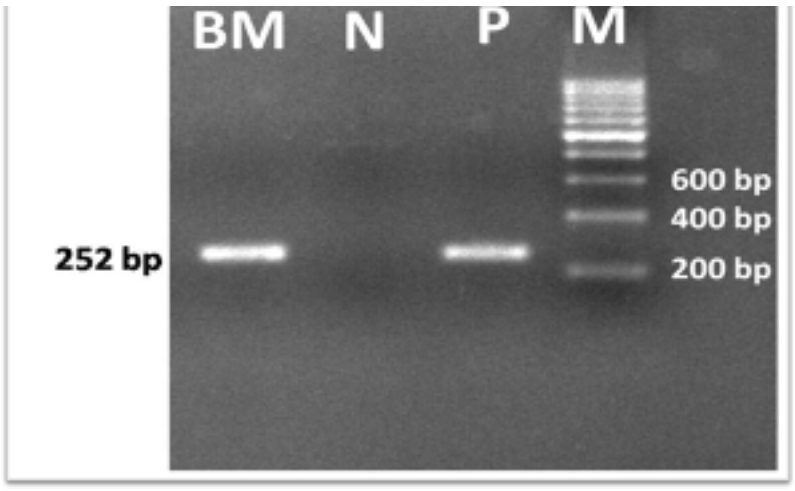

Figure 2. Agarose gel electrophoresis of PCR assay products. Lane M: 200 bp DNA ladder (ROTH); lanes BM: DNA of *Brucella melitensis* species from the aborted fetus (252 bp); lane P: positive control DNA of *Brucella melitensis* vaccine; lane N negative control.

Table 3. Prevalence of *Brucella melitensis* infection with risk factors in sheep and goats.

Factors	Levels	Sheep		Goats	
		Tested fetuses	(%) of infected fetuses	Tested fetuses	(%) of goats infected fetus
History of abortion	Yes	7	4(57.1%)	7	2(28.5)%
	No	46	13(28.2%)	13	10(7.6)%
Sex	Male	30	9(40.9%)	6	5(83.3)%
	Female	23	8(30.7%)	14	7(50)%
Age of fetus	≥3Months	10	3(33.3%)	5	1(20)%
	3-5Months	17	5(29.4%)	6	3(50)%
	>5Months	26	9(34.6%)	9	8(88.8)%
Age of dam	≥2years	16	5(31.2%)	9	2(22.2)%
	2-5 years	28	9(32.1%)	7	7(100)%
	>5years	9	3(33.3%)	4	3(75)%
Parity	First	15	7(46.6%)	8	6(75)%
	Second	19	4(21%)	5	3(60)%
	Third	10	2(20%)	4	1(25)%
	Fourth	9	4(44.4%)	3	2(66.6)%
Fetus organ	Spleen	53	4(7.5%)	20	4(20)%
	Liver	53	5(9.4%)	20	2(10)%
	Abomasa	53	8(15%)	20	6(30)%

Table 4: Multivariate logistic regression model and confidant interval (CI) for variable association with fetus contamination in 73 aborted sheep and goats.

Species	Infected fetus organ	Independent variable	Levels	Odds ratio	95% CI (Confidant Intervals)	P-value
Goats	Liver	History of abortion	Yes	2	1.8-11.2	<0.01
			No	Reference		
Sheep	Liver	History of abortion	Yes	3.5	0.8-1.1	<0.01
			No	Reference		
Sheep	liver	Fetus Sex	Male	1.006	1.1-42	0.6
			Female	Reference		
Goats	Abomasa	History of abortion	Yes	7	0.7-42	<0.01
			No	Reference		
Sheep	Spleen	History of abortion	Yes	5.7	0.1-16.5	0.24
			No	Reference		
Sheep	Spleen	Fetus sex	Male	1.3	0.1-16.5	1
			Female	Reference		
Goats	Spleen	Fetus ex	Male	1.3	0.1-16.5	1
			Female	Reference		

DISCUSSION

Abortion has one of the greatest economic loss impacts on animal production, especially in sheep and goats. One of the most common serious causes of parturient disturbance in small ruminants is brucellosis [23]. Numerous cases of brucellosis are found in sheep and goats in Sulaymaniyah province each year[24]. Therefore, this study aimed to detect the causative agents of abortions by culture and PCR technique.

There was no positive sample of both B. *abortus* and *B. suis,* this may due to the gap between bovine and swine species with sheep and goats in Sulaymaniyah province. The type of breeding system in Sulaymaniyah city is a separation between small ruminants and cattle that may cause mixed infection between the animal species.

Regarding the isolation of the *Brucella* by culture method, the best fetus organ was abomasal content. This is maybe because abomasums contain some chemicals that lead to chemo taxes and aggregation of the pathogen. The parity in this study was a very significant factor and most abortion was in the first parity. This explains the probability of the first infection by Brucella in the tested sheep and goats. Another factor that affects the infection by *Brucella* in sheep and goats is the age of the dam. The results show that age between 2-5 years was most susceptible to abortion and most of them were infected by brucellosis. At this age, the dam may expose to another pathogen or noninfectious factor that causing abortion.

Results of the present study revealed that *B. melitensis* was a significant causative agent of abortion in sheep and goats, with prevalence rates of (32.7%) and (60%) respectively. On the other hand, in another study, a considerable relation between gender and infection was recorded and this result was consistent with the findings of Mahdavi *et al* (2008) among sheep in Iran [25]. The aborted male sheep fetuses were higher than aborted female sheep fetuses, but in goats, female aborted fetuses were higher than male this high rate may be due to differences in the hormonal contents.

The presence of the same environmental and geographical characteristics and similar styles of animal breeding may be the main factors explaining the similarity of results for Iraq and Iran. Mohammadi *et al.* (2016)found that the prevalence of brucellosis in sheep was (17.5%) [26].

This difference may be due to some environmental factors.

Another study, performed on sheep and goats in Iran (2015-2016) by Shirazi *et al.,* demonstrated that the prevalence rate of brucellosis in aborted animals was seriously significant. This emphasizes that brucellosis has been a critical impetus for abortion in sheep and goats[27].

The PCR data revealed that all tested samples from aborted sheep and goat fetuses were positive for *Brucella* genus and all isolates were *B. melitensis*. Thus, the present results indicated that *B. melitensis* was the main common cause of abortion in sheep and goats and this broadly agrees with the results of [12]. Our results also reported that some aborted cases in sheep and goat were confirmed negative for brucellosis and this could be due to other bacterial and viral infections, and possibly due to elements deficiency. Among the numerous endemic infectious agents, *B. melitensis* remains one of the riskiest to animal production and public health in Iraq [28].

The independent variable (history of abortion) statistically shows a highly significant effect both in sheep and goats on the rate of abortion, which may be explained by the time of infection by *Brucella*.

In 2016, a researcher [29] detected *B. melitensis* in sheep in Sulaymaniyah province by using *OmP2a,* published in the NCBI database under accession number KP681853, and this agreed with the finding of the present study that *Brucella melitensis* is the most endemic and pathogenic species for abortion in sheep in Sulaymaniyah province.

In conclusion, *B. melitensis* is one of the common causes of abortion in sheep and goats in Sulaymaniyah Province. PCR assay is a specific and sensitive choice for the detection of *B. melitensis*.

For autopsy sampling, the abomasa were a popular target for isolation and detection of *B. melitensis*. A highly influential risk factor with the rate of infection was the history of abortion both in sheep and goats.

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